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14. ABSTRACT
Infiltrating peripheral nerve sheath tumors (PNST) are associated with significant neurological deficits and nerve damage. An initial aim of this project is to determine how tumor progression leads to loss of nerve function. A second aim is to determine if nerve damage caused by PNST is reversible and the potential for nerve regeneration after PNST eradication. Additional aims will test photodynamic therapy as modality for eradication of PNST without incurring substantial collateral damage to functioning nerve. To date we have mainly accomplished the first and second aims. Our work tested that hypothesis that tumor-induced nerve damage and loss of neurological function is reversible. Findings indicate that this is true only in the formative stages of tumor growth associated with low-level functional deficits. Tumor progression leads to increasing and permanent loss of nerve function. Function deficits persist even after tumor eradication. Results indicate that the tumor destroys the nerve, ablates supporting cells and replaces nerve structure with an impenetrable fibrotic mass. Even though afflicted neurons remain viable they fail to regrow axons into the fibrotic mass (even when devoid of tumor cells). The main conclusion of our findings is that tumor eradication does not induce or support nerve regeneration. Instead, a fibrotic mass remains that must be resolved for axonal regrowth to occur. This suggests that conventional therapies (e.g., radiation) may not provide for nerve regeneration and recovery of function. Our next objectives will provide a better understanding of the way PNST and therapies damage nerve. We will look much more closely at ways to resolve these obstacles and hopefully discover ways to allow for nerve regeneration when treating PNST.

15. SUBJECT TERMS
peripheral nerve sheath tumor, neurofibroma, photodynamic therapy, neurological deficit

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INTRODUCTION

Infiltrating peripheral nerve sheath tumors (PNST) are associated with significant neurological deficits and defy surgical removal without incurring further nerve damage. PNSTs often cause progressive demyelination, Schwann cell displacement and variable loss of supporting cells and axons within the tumor. An initial objective of this project is to determine how tumor progression leads to loss of nerve function. A second objective is to determine if nerve damage caused by infiltrating PNST is reversible and the potential for nerve regeneration after PNST eradication. Our studies will test the hypothesis that tumor-induced nerve damage and loss of neurological function is reversible. We will determine the potential for both spontaneous and experimentally induced nerve regeneration after tumor eradication. Photodynamic therapy (PDT) is a promising modality for the eradication of PNSTs. Our preliminary studies indicate that PDT effectively kills human NF1 tumor xenografts growing in mouse nerve. However, we found that highly tumoricidal PDT protocols targeted at normal nerves can cause significant nerve damage. Our preliminary studies show the neurological deficits incurred by PDT are transient and recovery of function occurs naturally in mice. We will test the hypothesis that PDT applied to infiltrating PNSTs can be highly tumoricidal without substantial permanent collateral damage to normal nerve.

BODY

OBJECTIVE 1: Determine the regenerative potential of nerves damaged by peripheral nerve sheath tumors.

Task 1: Examine PNST-induced damage to nerve: [months 1-6].

An initial goal of this project is to determine how tumor progression leads to loss of nerve function. A second main goal is to determine the preservation or loss of nerve elements and sheaths required for nerve regeneration. These experiments will determine if nerve damage caused by infiltrating peripheral nerve sheath tumor (PNST) is reversible and the potential for nerve regeneration after PNST eradication.

PROGRESS

NF1 tumor xenografts were initiated within the sciatic nerves of mice. Sciatic nerve mediated sensory and motor functions were scored weekly to monitor neurological deficits; these are indicative of tumor progression. A static sciatic function index (SSI) was calculated weekly. Tumor-bearing mice were terminated when their SSI indicated a functional deficit of 25%, 50% and 75%. Representative SSI plots are shown in Fig. 1. Engrafted nerves were removed for histological examinations. Tumor progression is shown in Fig. 2. Xenografted human tumor cells were identified by immunolabeling for huGST. Tumor growth was highly consistent and time-depended. Tumor progression correlated well with loss of neurological function.

Tumors were initiated within the tibial nerve branch of the sciatic nerve. In formative stages of PNST growth associated with 25% and 50% loss of function, tumor cells grew diffusely within the nerve core, infiltrating nerve elements but rarely spreading

to the adjacent peroneal nerve branch of the sciatic nerve (Fig. 2A-D). Nerve deterioration was apparent before substantial tumor expansion. Surprisingly, tumor growth did not simply displace nerve fibers, but appeared to consume them. Even when tumor cells were diffuse, their presence was associated with clearing of axons, myelin, Schwann cells and the surrounding endoneurial compartment (Fig. 3). This surely resulted in a progressive loss of nerve function. Laminin, a hallmark component of the endoneurial basal lamina, was absent from areas occupied by tumor cells. The extracellular matrix of nerve was replaced with a fibrotic matrix rich in chondroitin sulfate proteoglycan.

Continued tumor growth was associated with a progressive loss of nerve elements and neurological function. In mice with ≥75% loss of function, large tumors were found that also spread to adjacent nerve branches and occupied the entire sciatic nerve (Fig. 2E, F). The majority of nerve elements within the tumor mass were gone. Tumor cellularity was high and the expanding mass greatly increased nerve diameter. In most cases, some bundles of axons remained. These were found only at the extreme perimeter of the tumor mass, outside the tumor capsule and likely highly compressed. Nevertheless, massive tumors were associated with severe loss of neurological function. In the nerve distal to the tumor, Wallerian degeneration (hypercellularity, myelin fragmentation, axonal swelling) was widespread, indicative of tumor-induced axotomy.

Neuronal cell death was assessed by routine histology and FluoroJade staining in the dorsal root ganglia and ventral motor neurons (at L4-5). Interestingly, there were no indications of neuronal cell death associated with the tumor-induced axotomy. Based on this observation, there remained a potential for axonal regeneration. Axotomy resulting from traumatic injury or toxic lesion causes a substantial axon sprouting response. Oddly, we found no evidence of axonal sprouting in the tumor vicinity. Our conclusion is that PNST occupancy results in the loss of nerve elements and, therefore, loss nerve conduction through the tumor. PNST replaces the nerve matrix and therein appears to prevent axonal sprouting, despite the continued survival of the neuronal cell bodies.

Task 2: Evaluate nerve regeneration after immunorejection of PNST: [months 3-12]

Invasive PNSTs remodel nerve and may disrupt nerve sheath continuity that guides axonal regrowth and appropriate reinnervation of end organs. In many tumors nerve fascicles, although displaced, persist along with intact endoneurial basal laminae encasing axon-Schwann units. There is good reason to believe that these fibers are functional and retain full regenerative capacity in the event of axotomy. On the other hand, axonal degeneration is associated with tumor progression. It is unknown to what extent infiltrating PNSTs cause neuronal loss, permanent nerve damage or affect the potential for axonal regeneration. In this Aim we determined the capacity for nerve regeneration after infiltrating PNST is eradicated. At various stages of PNST progression, xenogeneic tumors were eliminated by restoring immunorejection competency to the immunodeficient scid mouse host. After PNST rejection, nerve regeneration and recovery of neurological function were evaluated in two conditions. One condition examined spontaneous nerve regeneration. The second condition induced regeneration by proximal axonotmesis (crush) injury. These experiments tested if nerve damage caused by infiltrating PNST is reversible and examined the potential for nerve regeneration after PNST eradication.

PROGRESS

This aim required reconstitution of tumor immunorejection in the immunodeficient xenograft hosts. The goal was to provide the host mouse with the ability to develop an immunoresponse that eradicated the xenograft tumor. This was achieved by injecting the immunodeficient (scid) mice with wild-type mouse bone marrow cells. De novo production of serum IgM was monitored as an indication of immunoefficiency. All scid mice injected intravenously with wild-type bone marrow cells showed a rapid and progressive increase in serum IgM titer, reaching 50% of wild-type levels after 2-3 weeks.

Next, intraneural PNSTs were initiated in mouse sciatic nerves. Sciatic nerve mediated sensory and motor functions were scored weekly and SSIs calculated. Immunorejection was reconstituted in tumor-bearing mice with varying stages of functional deficit (indicative of tumor progression). From previous work we know that tumor progression beyond 10 weeks presents a terminal health risk. Despite the initial nerve function deficits, all mice became stable after immunoreconstitution. Function deficits plateaued and all mice remained viable. At termination dates 5-15 weeks after immunoreconstitution, the tumor-engrafted nerves were examined for tumor cell death and nerve cell viability. All tumors showed pervasive cell death. All indications were that immunoreconstitution eradicated the tumors, or nearly so. While tumor cells were killed a dense, fibrotic mass remained. Mice with a low loss of function showed slight recovery of function. Importantly, mice with major loss of function due to tumor occupancy recovered little lost function. Results indicate that little spontaneous nerve regeneration occurred after tumor eradication. Instead, it appeared that the fibrotic tumor tissue might prevent nerve regeneration and recovery of function.

A second set of tumor-bearing mice were established in the same conditions and evaluated as described above. As above, these mice (with various levels of functional deficit) were injected with wild-type bone marrow cells reconstitution immunorejection for tumor eradication. Next, axonal regeneration was induced in all axons proximal to the tumor by a nerve crush injury. Here, tumor was eliminated (rejected) and then axons were induced to regrow proximal to the tumor lesion. In several mice with small tumors and low loss of function, stimulation of nerve regrowth by crush injury resulted in better recovery of function than without the nerve crush. However, mice with large tumors, high functional deficits and dominant fibrotic masses showed no signs of recovery of function. End-point histology corroborated this finding. No axonal regeneration was observed within the acellular tumor mass. A detailed examination of the deleterious, residual fibrotic tumor mass is underway. The main conclusion of our findings is that tumor eradication does not induce or support nerve regeneration. Instead, a fibrotic mass remains that must be resolved for axonal regrowth to occur.

OBJECTIVES FOR THE NEXT TWO YEARS

No changes in the Objectives are anticipated in the second and third years of this project.

OBJECTIVE 2: Determine the mechanisms and pharmacodynamics of PDT induced nerve damage.

Task 1: Test various drug-light intervals in PDT applied to normal nerve and evaluate nerve damage: [months 12-16]

Task 2: Test various drug-light intervals in PDT applied to normal nerve and evaluate nerve regeneration: [months 14-20]

OBJECTIVE 3: Determine the tumoricidal effects of LS11-PDT applied to intraneural tumor xenografts.

Task 1: Test various drug-light intervals in PDT applied to xenograft tumors and evaluate tumor kill: [months 20-24].

Task 2: Determine the most tumoricidal mode of PDT, vascular versus cell targeting: [months 20-26].

OBJECTIVE 4: Determine the regenerative potential of nerves after tumoricidal PDT treatment.

Task 1: Evaluate long-term regression of nerve sheath tumor xenografts after PDT: [months 26-36].

Task 2: Evaluate long-term effects of tumoricidal PDT on nerve regeneration and recovery of function: [months 26-36].

KEY RESEARCH ACCOMPLISHMENTS

1. Reliable initiation of PNST xenografts in mouse nerve
2. Gauge tumor growth by monitoring loss of nerve function
3. Established digital video techniques to score loss of sciatic nerve function in mice
4. Establish methods to reconstitute immunocompetency in immunodeficient mice
5. Prove reconstitute of immunocompetency is an effective noninvasive means to eliminate PNST xenografts
6. Determined PNST ablate nerve fibers and sheaths
7. Determined PNST form an extracellular matrix barrier that prevents axon regrowth and nerve regeneration

REPORTABLE OUTCOMES

Animal model for tumor eradication by reconstitution of immunocompetency (manuscript in preparation)

CONCLUSION

Our work tested that hypothesis that tumor-induced nerve damage and loss of neurological function is reversible. Findings indicate that this is true only in the formative stages of tumor growth associated with low-level functional deficits. Tumor progression leads to increasing and permanent loss of nerve function. Function deficits persist even after tumor eradication. Results indicate that the tumor destroys the nerve, ablates supporting cells and replaces nerve structure with an impenetrable fibrotic mass. We found that the neurons remain viable but fail to regrow axons into the fibrotic mass (even when devoid of tumor cells). This has several implications for managing PNST. Most often the treatment of PNST involves a "wait and see approach" when symptoms are minor and there is little loss of function. This approach is contraindicated by our findings. More disturbing is that substantial tumor damage to nerve may not be reversible even after effective tumor eradication. . The main conclusion of our findings is that tumor eradication does not induce or support nerve regeneration. Instead, a fibrotic mass remains that must be resolved for axonal regrowth to occur. This suggests that conventional therapies (e.g., radiation) may not provide for nerve regeneration and recovery of function. An alternative and more aggressive approach is surgical removal of the tumor-laden nerve and restoring nerve continuity with a graft. Nevertheless, the next objectives of this project will provide a better understanding of the way PNST and therapies damage nerve. We will look much more closely at ways to resolve these obstacles and hopefully discover ways to allow for nerve regeneration when treating PNST.

SUPPORTING DATA

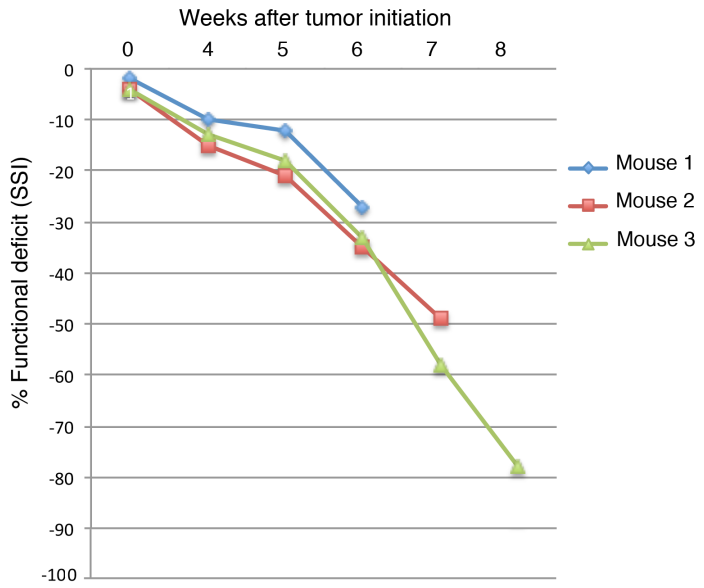

Figure 1. Representative plots of sciatic nerve function in mice after PNST tumor xenografting. The footspread of ambulating mice was monitored by videography. A static sciatic nerve function index (SSI) was calculated from semi-automated digital measurements of toespread. The SSI represents a measure of function deficit (negative deflection from normal = 0). The SSI (deficit) correlated with PNST progression.

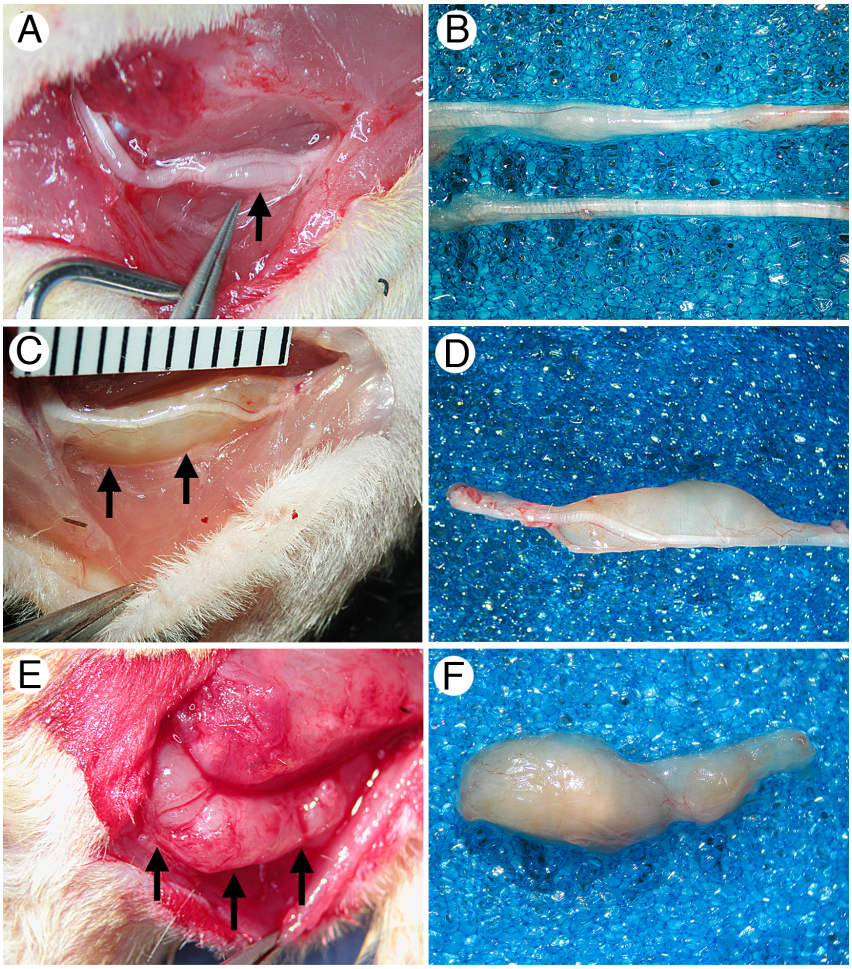

Figure 2. Representative gross appearance of PNST xenografts. Tumors were initiated in a branch of the sciatic nerve. Initially (4-5 weeks) tumors (arrow) caused a slight swelling of the nerve (A,B). Normal nerve is shown as the lower image in B. This was associated with ≈25% function deficits. A sizable tumor mass (arrows) was seen after ≈7 weeks (C,D). An unaffected nerve branch is evident. (The ruler has 1 mm increments). This tumor stage was associated with ≈50% function deficits. Massive tumors (arrows) occurred after 8 weeks that spread to and occupied both branches of the sciatic nerve (E,F). These tumors resulted in severe loss of function and eventually failing animal health.

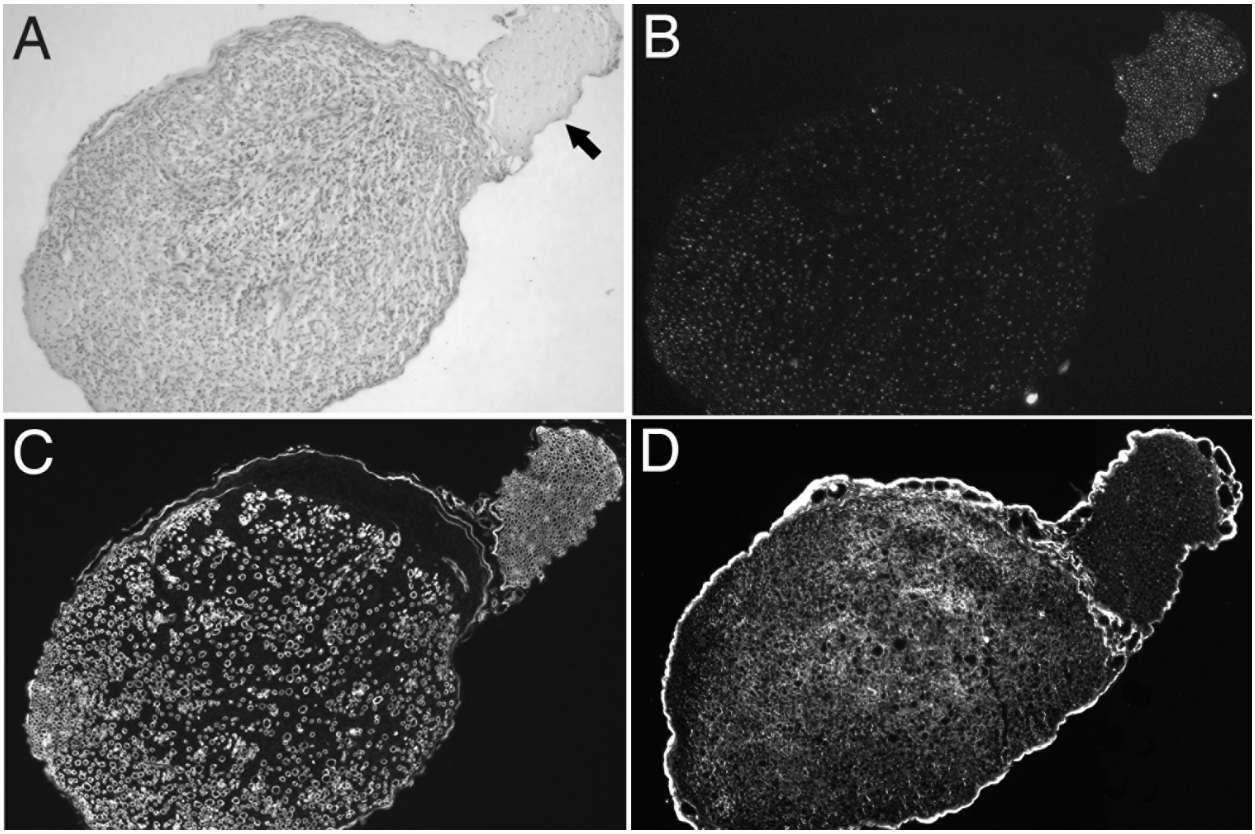

Figure 3. Histology of early stage (5 weeks) PNST xenograft and nerve damage. Immunostaining with the human specific GST antibody shows the diffuse infiltration and moderate density of PNST cells (A). The low cellularity of an unaffected small nerve branch is evident (arrow). Immunolabeling for neurofilament in axons indicates nerve organization is disrupted by the infiltrating tumor (compare the tightly packed axons in the unaffected branch)(B). Laminin immunolabeling clearly shows the disruption of the nerve sheaths and basal lamina surrounding the axons (C). Immunolabeling for chondroitin sulfate protoeglycan demonstrates the formation of a fibrotic matrix by the PNST (D).

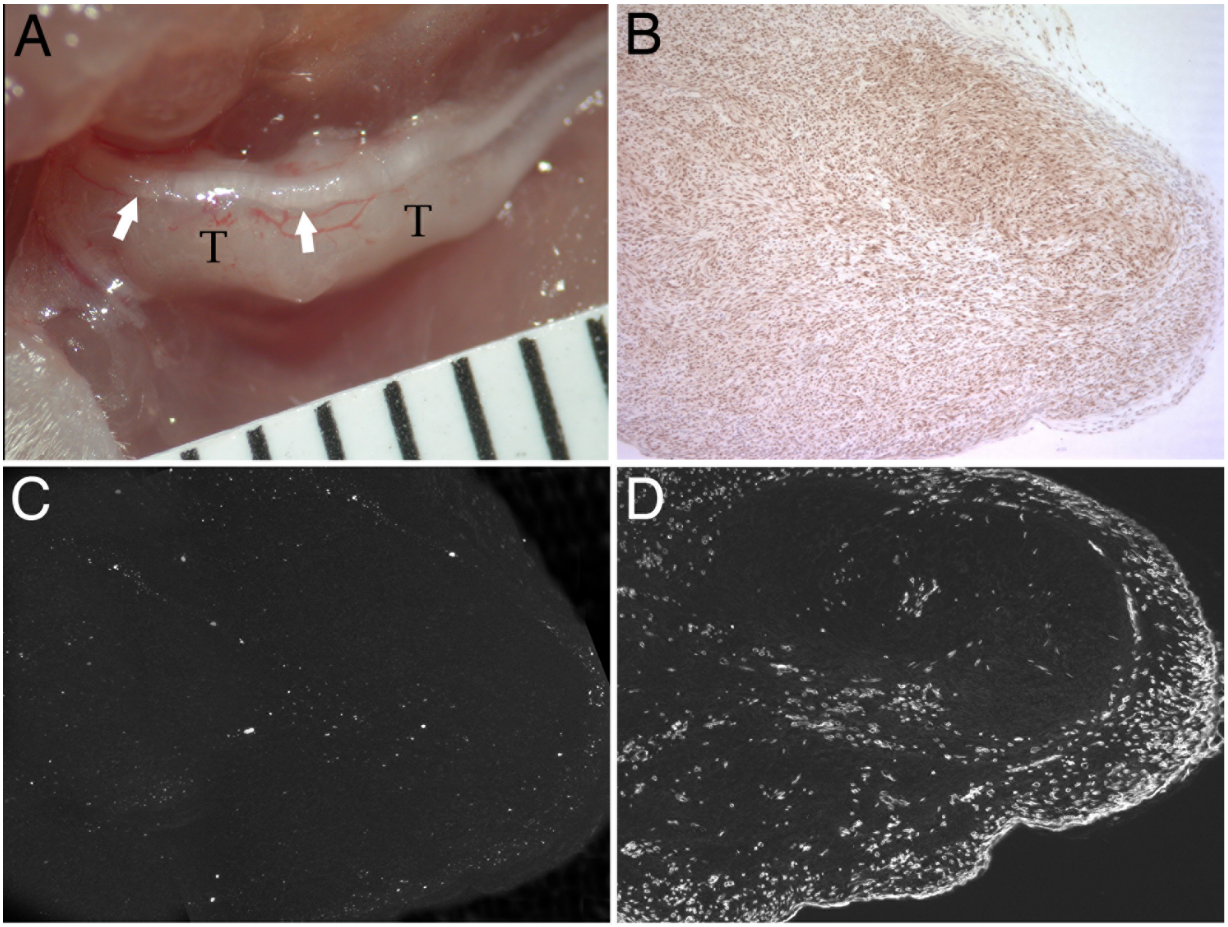

Figure 4. Histology of middle stage (7 weeks) PNST xenograft and nerve damage. Tumor (T) has fully occupied the tibial nerve branch and is starting to spread to the adjacent peroneal branch (arrows) (A). (Ruler increments = 1 mm) Immunostaining with the antibody specific for human GST shows hypercellularity and pervasive tumor occupancy (B). Immunolabeling for neurofilament indicates widespread loss of axons within the tumor (C). Immunolabeling for laminin indicates the nerve sheaths are disintegrated as well (D).